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Molecular Mechanisms Involved in Adenosine-Induced Endothelial Cell Barrier Enhancement

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Abstract

Extracellular adenosine is a physiologically relevant agonist released by various sources, including endothelial cells (EC) and activated platelets, with complex effects mediated via activation of P1 purinergic receptors. Adenosine-induced EC production of glutathione peroxidase 1 and nitric oxide is recognized, and an anti-inflammatory mechanism has been described. Effects of extracellular adenosine on the pulmonary EC barrier function and vascular permeability, however, remain poorly characterized. In this study, we demonstrated the adenosine-induced rapid dose-dependent barrier enhancement in human pulmonary artery EC (HPAEC) as measured by an increase in transendothelial electrical resistance (TER). We have shown that HPAEC express only A2A and A2B adenosine receptors. Pharmacological and siRNA depletion studies indicate that A2A, but not A2B receptor activation is required for the adenosine-induced TER increase. Depletion of Gαs with a specific siRNA significantly attenuated the adenosine-induced TER response in HPAEC. In contrast, depletion of either Gαq or Gαi2 did not affect the adenosine-induced TER increase. This suggests that the adenosine-induced TER increase is cAMP-dependent. The adenosine-induced barrier enhancement effects were associated with a rearrangement of the EC F-actin component of the cytoskeleton, enhanced cell surface presentation of cell-cell junctional protein VE-cadherin and an involvement of Myosin-light-chain phosphatase (MLCP). Our results suggest, for the first time, that adenosine regulates the EC barrier function via A2A receptors followed by Gαs engagement and is associated with cytoskeletal activation.

Keywords

myosin-light-chain phosphatase; anti-inflammation; P1 receptor agonists; human pulmonary artery endothelial cells

1. Introduction

A major function of the vascular endothelial cell (EC) monolayer is to serve as a selective barrier to fluid and solute flux across the blood vessel wall. It is known that loss of this function results in tissue inflammation, the hallmark of inflammatory diseases such as acute respiratory distress syndrome (ARDS). Less information is available about the mechanisms

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of EC barrier maintenance and protection. There are comprehensive reviews dealing with the general subject of endothelial permeability discussed from different perspectives (Mickell and Curry, 1999; Tuma and Hubbard, 2003). Loss of barrier function results from the opening of gaps between adjacent cells as a consequence of both loss of cell adhesion and activation of the endothelial contractile machinery (Michel and Curry, 1999). The generation of contractile forces by EC can cause adjacent cells to retract from each other (Schnittler et al, 1990). During the past decade, many efforts have been undertaken to analyze the mechanisms that activate the contractile machinery, such as those provoked by inflammatory mediators like thrombin (Essler et al, 1998). Recent studies demonstrate the therapeutic potential of purinergic agonists and antagonists for the treatment of cardiovascular and pulmonary diseases (Burnstock, 2003; Polosa and Holgate, 2006; Brown et al, 2008).

Adenosine, a purine nucleoside, is produced in response to metabolic stress and cell damage, and elevations in extracellular adenosine are found in conditions of ischemia, hypoxia, inflammation, and trauma (Hasko and Cronstein, 2004). The dominant pathway leading to high extracellular adenosine levels during metabolic stress is the release of precursors and adenine nucleotides (mostly ATP) from the cell, followed by the extracellular catabolism of nucleotides to adenosine by a cascade of ectonucleotidases (Zimmermann, 2000; Linden, 2001; Eltzschig et al, 2004; Thompson et al, 2004). Adenosine mediates its functions through activation of specific G-protein coupled receptors, for which four subtypes have been identified (A1, A2A, A2B, and A3) (Fredholm et al, 2005). Adenosine can activate A1, A2A, and A3 receptors with EC₅₀ in the 0.2–0.7 μ M range, whereas the potency of adenosine at A2B receptors is lower (EC₅₀: 24 μ M) (Fredholm et al, 2001). The physiological adenosine concentrations rarely exceed 1 μ M, and therefore, physiological levels can activate A1, A2A and A3 receptors, whereas A2B receptor activation requires pathophysiological conditions. A1 and A3 receptors (coupled to Gai/o G-proteins) activation results in decreased intracellular cAMP levels. Both A2A and A2B receptors couple to Gas proteins and stimulate adenylate cyclase, but only A2B stimulates phospholipase C β through coupling to Gq proteins leading to the activation of protein kinase C and calcium mobilization. This receptor complexity reflects the multifaceted role that adenosine has in health and disease. In general, the A2-type receptors have been described as sensors of tissue damage during an immune response (Sitkovsky and Ohta, 2005), and A2A receptors have been described as having a non-redundant role in the attenuation of inflammation and tissue damage *in vivo* (Ohta and Sitkovsky, 2001). Indirect evidence suggests that the adenylate cyclase stimulatory A2B adenosine receptor also affects vascular function (Dubey et al, 2000; Gao et al, 1999). Adenosine receptors play a major role in several inflammation-associated processes by inhibiting immune activation and preventing excessive tissue damage. This has been demonstrated by using A2A receptor-null mice (Dubey et al, 2000).

In previous studies, we have demonstrated that extracellular ATP stabilizes the barrier function of the pulmonary endothelial monolayers (Kolosova et al, 2005). Since, extracellular ATP rapidly degrades into adenosine from a coordinated two-step enzymatic conversion of precursor nucleotides via the ecto-apyrase (CD39) and the ecto-5'-nucleotidase (CD73), we hypothesized that adenosine could also be involved in EC barrier function via P1 receptors. Thus, determining the adenosine receptor expression and understanding extracellular adenosine signaling in pulmonary EC would shed some light on the role of adenosine receptors, particularly in acute lung injury when levels of extracellular adenosine are substantially elevated due to increased expression of CD39 and CD73 (Eckle et al, 2007; Reutershan et al, 2009).

2. Material and Methods

2.1. Materials

All the reagents were obtained from SIGMA-Aldrich (St. Louis, MO) unless otherwise indicated. Monoclonal VE-cadherin antibodies were purchased from BD Biosciences (San Diego, CA). MYPT1 polyclonal antibodies were obtained from Upstate Biotechnology (Charlottesville, VA). Texas Red-conjugated phalloidin, and goat antimouse Alexa 488 antibodies were purchased from Molecular Probes (Eugene, OR). All the small interfering RNA's (siRNAs) were purchased either from Ambion (Austin, TX) or Dharmacon (Chicago, IL). siPORT Amine transfection reagent was obtained from Ambion (Austin, TX). Trizol was obtained from Invitrogen (Carlsbad, CA). Adenosine agonists and antagonists were obtained from Tocris (Ellisville, MO). Neutravidin-agarose and sulfo-NHS-LC-biotin were purchased from Pierce Chemical (Rockford, IL).

2.2. Cell Culture

Human pulmonary artery EC (HPAEC) and EBM-2 complete medium were purchased from Lonza (Allendale, NJ) and the cells were cultured according to the manufacturer protocol. HPAEC were utilized at early passages (5–10) (Kolosova et al, 2005).

2.3. Depletion of endogenous mRNA using siRNA approach

To reduce the mRNA content of endogenous A2A, A2B, and MYPT1, HPAEC were treated with specific siRNA duplexes, which guide sequence-specific degradation of the homologous mRNA. Non-specific scrambled siRNAs were used as a control treatment. HPAEC were plated on 60 mm dishes to yield 60–70% confluence, and transfection of siRNAs was performed using siPORT Amine transfection reagent according to the manufacturer's protocol. Briefly, cells were serum starved for 1 hr followed by incubation with 20 nM of target siRNA (or scrambled siRNA) for 6 hrs in serum-free media. Then media with serum was added (1% serum final concentration) for 42 hrs before biochemical experiments and/or functional assays were conducted. To estimate the efficiency of mRNA depletion, forty-eight hours later, cells were lysed in Trizol and specific mRNA depletion was analyzed by RT-PCR. For transendothelial resistance measurement, cells were plated to yield 60–70% confluence in electrode wells and transfected with siRNA as described (Kolosova et al, 2005).

2.4. Measurement of endothelial monolayer resistance

The barrier properties of EC monolayers were characterized using highly sensitive electrical cell-substrate impedance sensing (ECIS) instrument as described previously (Takizawa et al, 2003; Kolosova et al, 2005). TER data were normalized to the initial voltage.

2.5. Immunofluorescent microscopy

Immunostaining was performed as described previously (Kolosova et al, 2005). Specific antibodies were used for detection of VE-cadherin. The DNA-binding, fluorescent dye 7-amino-actinomycin D (7AAD) was used to stain cell nuclei (Rabinovitch et al, 1986). The percentage of the total cell surface area occupied by the VE-cadherin labeled cell-cell junctions was quantitatively determined using the ImageJ software available from the National Institute of Health web site (<http://rsb.info.nih.gov/ij/download.html>).

2.6. Cell-surface biotinylation

The cell-surface presentation of VE-cadherin was analyzed as described previously for other extracellular and transmembrane proteins (Janiak, et al., 2006). Briefly, adherent HPAEC were labeled for 15 min with 0.5mg/ml cell-impermeable sulfo-NHS-LC-biotin in PBS (pH

7.4) on ice and the biotinylation reaction was quenched with TBS, pH 7.5 for 10 min on ice. Then, the cells were lysed in 1 ml of TBS containing 1% SDS and boiled for 5 min. After clearing the cell extracts by centrifugation for 20 min at 15,000 rpm, the supernatants were incubated with 100 μ l of neutravidin-agarose beads to isolate biotinylated (cell-surface) proteins. Biotinylated VE-cadherin bound to the beads was then analyzed by 10% SDS-PAGE and immunoblotting with respective antibody. Quantification data was obtained using NIH Image 1.63f software.

2.7. Adenosine receptor agonists and antagonists study

HPAEC were challenged with agonists specific for adenosine receptors (A2A and A2B) and the TER was registered. In other experiments, the HPAEC were pretreated with the A2A or A2B receptor specific antagonists for 30 min, then challenged with adenosine and TER was registered to examine the barrier enhancement induced by adenosine in the presence or absence of the antagonists.

2.8. Statistical analysis

All measurements are presented as the mean $\pm$ SEM of at least 3 independent experiments. To compare results between groups, a 2-sample Student t test was used. For comparison among groups, 1-way ANOVA was performed. Differences were considered statistically significant at $p < 0.05$. We routinely use this analysis in our studies (Kolosova et al, 2005).

3. Results

3.1. Adenosine increases Transendothelial Electrical Resistance

Adenosine increases the TER of HPAEC monolayers in a dose-dependent manner (Fig. 1A) indicating barrier enhancement. Importantly, simultaneous addition of thrombin and adenosine or addition of adenosine after thrombin significantly attenuated the thrombin-induced EC permeability demonstrating the barrier-protective effects of adenosine (Fig. 1B).

3.2. Adenosine affects cell-cell junctions

Immunofluorescence studies demonstrated changes in distribution of cell-cell junctional proteins after adenosine treatments. VE-cadherin, a major component of the endothelial junctions, was more pronounced at the cellular periphery, presumably at cell-cell contacts (Fig. 2A). The calculated percentage of total cell surface area (Kolosova et al, 2005) occupied by VE-cadherin-labeled cell-cell junctions confirmed that adenosine-induced a significant increase in the surface area of cell-cell interfaces as a percentage of total cell surface area (Fig. 2B). Using another approach (cell-surface biotinylation), we were able to demonstrate time-course of cell surface expression of VE-cadherin and cellular redistribution of the protein: while total levels of the protein remained the same, 30 min. treatment with adenosine led to dramatic increase in cell-surface VE-cadherin (up to 3 times) (Fig. 2C). Interestingly, cell-surface elevation of VE-cadherin was a very early event in adenosine-mediated cell response and could be detected after at least 5 min. of the treatment. Taken together, our data (Fig. 2) suggest the involvement of VE-cadherin in adenosine-mediated EC barrier enhancement.

3.3. Adenosine induces EC cortical cytoskeletal rearrangement

The EC cytoskeleton (primarily actin filaments and microtubules) is a critical determinant of vascular integrity and barrier regulation (Bogatcheva and Verin, 2008, Dudek et al, 2004). Therefore, first we examined the involvement of the F-actin component of the cytoskeleton in adenosine-induced EC barrier enhancement. We pretreated the HPAEC monolayers with the actin-depolymerizing agent cytochalasin B, which produced a prompt decline in TER

(Fig. 3A). Unlike the protective response to adenosine challenge after established thrombin-induced barrier dysfunction (Fig. 1B), adenosine failed to increase TER when added after cytochalasin B challenge, indicating a critical requirement for dynamic cytoskeletal rearrangement and an intact actin part of cytoskeleton for adenosine barrier-enhancing effects (Fig. 3A). Secondly, we employed a model of microtubule disruption with nocodazole (an anti-neoplastic agent which exerts its effect directly by depolymerizing microtubules). Fig. 3B demonstrates that nocodazole failed to alter the adenosine-mediated TER response, indicating the specific requirement for actin dynamic assembly/disassembly in the adenosine mediated barrier-enhancing response.

3.4. Expression of adenosine receptors in HPAEC and the effect of pharmacological agonists and antagonists on the TER

Among the four adenosine receptors (A1, A2A, A2B and A3), HPAEC express only A2A (high affinity) and A2B (low affinity) receptors (Fig. 4A). Our quantitative Real-Time RT-PCR (qPCR) data indicate that A2B receptors mRNA expression was about three fold higher compared to A2A receptors. These data may also suggest a difference in the protein expression of the receptors. Since adenosine showed strong protective effects in thrombin induced EC dysfunction, we were interested in identifying the specific adenosine receptor that mediated this protective effect. We conducted a pharmacological approach using receptor specific agonists and antagonists. Treatment of HPAEC with the A2A receptor agonist p-DITC showed significant increase in TER (Fig. 4B) and that persisted for several hours. In addition, pretreatment with A2A the receptor antagonists, ZM241385 and CGS15943 significantly attenuated the adenosine-induced increase in TER (Fig. 4C). However, pretreatment with the A2B receptor specific antagonist, PSB 36, did not alter the adenosine-induced TER response (Fig. 4D). Together, the pharmacological studies suggest the involvement of A2A, not A2B receptors in an adenosine-induced TER response in HPAEC monolayers.

3.5. Effect of adenosine receptors (A2A and A2B) depletion on the adenosine-induced TER increase in HPAEC

To confirm the results obtained from pharmacological studies for the role of A2A receptors in an adenosine-induced EC TER increase, in this study, we individually depleted A2A and A2B receptors with the receptor specific siRNA in HPAEC. Depletion of A2A, but not A2B receptors significantly attenuated the adenosine-induced increase in TER (Fig. 5) indicating the involvement of A2A receptors in adenosine-induced EC TER increase. The control siRNA with scrambled sequence failed to attenuate the adenosine-induced TER increase thus demonstrating the sequence-specific depletion.

3.6. Adenosine response in mediated by Gas G-protein

To determine the role of trimeric G-proteins in adenosine-induced TER increase, HPAEC were treated with siRNAs specific for α -subunits of several trimeric G-proteins. Depletion of G α s with specific siRNAs significantly attenuated the increased TER induced by adenosine (Fig. 6). In contrast, depletion of either G α q or G α i2 did not to attenuate the adenosine-induced increase in EC TER (Fig. 6). These findings delineate the involvement of G α s in adenosine-induced EC TER increase.

3.7. Role of Ser/Thr protein phosphatases in adenosine-induced EC TER increase

There is a growing body of evidence that myosin-light-chain (MLC) kinase (MLCK) and phosphatase (MLCP) participate in the regulation of endothelial barrier function (Kolosova et al, 2005; Satpathy et al, 2005; Hartel FV et al, 2007). Conventional MLCP consist of a catalytic protein phosphatase 1 (PP1c) subunit, a myosin phosphatase targeting subunit

(MYPT1), and a smaller M20 subunit, not always present in all the phosphatase complexes (Tar et al, 2006, Hartshorne et al, 1998; Takizawa et al, 2003; Ito et al, 2004). MYPT subunit is essential for myosin phosphatase activity (Gailly et al, 1996; Johnson et al, 1996). Therefore, we used the MYPT1 siRNA to determine the role of MLCP in an adenosine-induced EC TER increase. Depletion of MYPT1 subunit of MLCP with siRNA significantly attenuated the adenosine-induced TER (Fig. 7A) which clearly demonstrates the involvement of MLCP. Our recent studies also have revealed the significance of protein phosphatase 2A (PP2A) in maintaining the EC cytoskeletal organization and barrier function (Tar et al, 2006, 2004). Pretreatment of HPAEC with PP2A inhibitor Fostriecin for 30 min failed to inhibit the adenosine-induced HPAEC TER increase (Fig. 7B) and confirms that PP2A is not involved in adenosine mediated effect.

4. Discussion

The vascular endothelium lining the intima of the blood vessels regulates a variety of functions. The maintenance by the endothelium of a semi-permeable barrier is particularly important in controlling the passage of macromolecules and fluid between the blood and interstitial space. It is known that loss of this function results in tissue inflammation, which is the hallmark of inflammatory diseases such as ARDS. We reported recently that ATP is a potent endothelial monolayer stabilizing agent via its P2Y receptors (Kolossova et al, 2005; Kolossova et al, 2008). Endothelium is a rich source of adenosine as a result of both the bidirectional flux of the nucleoside and its formation from ATP by endothelial exonucleotidases. Adenosine is a potent anti-inflammatory agent and plays a major role in cardiovascular protective mechanisms.

Our results demonstrate that adenosine is a potent and direct endothelial monolayer-stabilizing agent that can reverse barrier disruption induced by edemagenic agents such as thrombin (Fig. 1A and 1B). In addition, adenosine initiated the recruitment of VE-cadherin to the cell periphery as evidenced by either immunofluorescence or by biotinylation of cell-surface proteins suggesting the tightening of cell-cell contacts (Fig. 2). We were able to obtain information regarding the crucial steps of signaling mechanisms by which adenosine accomplishes barrier protection and to identify potential downstream mediators, since these can be useful therapeutic targets in the regulation of vascular leak and barrier dysfunction. Our results show that the cytochalasin B treated HPAEC failed to increase the TER upon adenosine treatment thus demonstrating the critical role of polymerized actin in EC barrier enhancement. In contrast, adenosine is able to reverse the effect of the microtubule inhibitor, nocodazole, on TER. The data strongly suggest the critical role of active cytoskeletal actin rearrangement in EC barrier protection induced by adenosine. Thus, the mechanism of adenosine-mediated EC barrier enhancement is similar to that described for the PKA agonists (forskolin, dibutyryl-cAMP etc.) by Stelzner et al, 1989. The treatment of EC with PKA-activating agents was accompanied by a rapid increase in polymerized actin, an increase in cell surface area, a loss of stress fibers, and increased phosphorylation of intermediate filaments (Stelzner et al, 1989).

Several studies have demonstrated that the adenosine receptors (A1, A2A, A2B, and A3) are widely distributed in pulmonary endothelium (Eckle et al, 2008; Wilson and Batra 2002; Balestrieri et al, 1998). Previous studies have indicated the role of adenosine in the platelet-mediated reduction in pulmonary vascular permeability (Paty et al, 1992) and bovine fetal aortic endothelial monolayer permeability (Haselton et al, 1993). According to our data (Fig. 4A), only A2A and A2B adenosine receptors are expressed in human pulmonary EC and A2A, but not A2B receptors are responsible for the adenosine-mediated EC barrier enhancement.

Adenosine is typically cleared from the extracellular environment by conversion to inosine via the action of adenosine deaminase, or by uptake into the cells through nucleoside transporters. Therefore, it is quite possible that the extracellular adenosine concentration is critical in receptor activation and subsequent signaling events. Allen-Gipson et al, 2007 showed that the A2A receptor mediated adenosine-stimulated epithelial cell wound healing is by the activation of cAMP-dependent PKA although all four adenosine receptors were expressed in the bronchial epithelial cells used for their study. Since migration of neighboring cells into the injury is important for rapid repair of damaged airway epithelium, therefore, it is quite possible that similar signaling mechanisms might be involved in pulmonary EC barrier enhancement via A2A receptors.

Depletion studies clearly demonstrate the principle involvement of A2A receptors in HPAEC TER increase and protection against thrombin-induced EC permeability decrease (Fig. 4 and 5). Consistent with the present study, other investigations indicate protective effects of adenosine on the pulmonary integrity via A2A receptors. For example, LPS-induced ALI was aggravated in A2A receptor null mice (Thiel et al, 2005). Moreover, genetic removal of the A2A adenosine receptor enhances pulmonary inflammation, mucin production, and angiogenesis (Mohsenin et al, 2007) and targeted deletion of A2A adenosine receptors attenuates the protective effects of myocardial postconditioning (Morrison et al, 2007).

MLCP is a major phosphatase responsible for the dephosphorylation of the regulatory light chain of myosin II. MYPT1 is responsible for activation and regulation of MLCP. Binding of PP1c to the N-terminal region of MYPT1 produces a 15-fold increase in catalytic activity and a 10-fold higher affinity for phosphorylated myosin. We and others have shown that Rho kinase mediated MYPT phosphorylation is tightly linked to agonist-induced EC contraction and permeability (Birukova et al, 2004; Kimura et al, 1996; Feng et al, 1999). Our data (Fig. 7A) suggest that adenosine induces MLCP activation since depletion of the regulatory subunit MYPT1 by siRNA attenuated the adenosine-induced barrier enhancement.

The activation of G α s-coupled A2A receptors in a variety of cell types leads to inhibition of pro-inflammatory pathways (Liou et al, 2008; Hasko and Pacher 2008; Kolachala et al, 2008; Palmer and Trevethick 2008; Garcia et al, 2008). Our siRNA based gene silencing data for several trimeric G-proteins demonstrate that depletion of the α subunit of the Gs trimeric G protein significantly attenuates the TER response induced by adenosine. In contrast, depletion of either G α q or G α i does not attenuate the adenosine-induced increase in TER (Fig. 5). This data indicates the involvement of only the G α s in barrier enhancement produced by adenosine. The mechanism of the G α s-protein function is well-documented (Hamm and Glichrst, 1996; Pierce et al, 2002). The initial step of G α s-mediated signaling is a direct interaction of activated G α s with the adenylate cyclase and stimulation of cAMP synthesis (Plagge et al, 2008). Our data suggest that the adenosine-mediated TER increase in HPAEC is cAMP-dependent. These results, however, differ from our previous data for the ATP-stimulated EC TER increase which elucidated the involvement of G α i2 and G α q without cAMP synthesis (Kolosova et al, 2008). Thus our present data and the previously published results (Kolosova et al, 2008) establish the collective but independent role of both P1 (adenosine) and P2Y (ATP) receptors in pulmonary EC barrier enhancement.

In summary, our data demonstrate the contribution of extracellular adenosine to maintain the integrity of pulmonary endothelium. While our detailed investigations of cell signaling in adenosine-induced EC barrier enhancement are in progress, they remain secondary to the main finding of this study that extracellular adenosine has a dramatic effect on the permeability function of EC. Adenosine-mediated TER increase is primarily dependent on signaling pathways affecting dynamic changes of cytoskeletal F-actin. Our results

demonstrate that HPAEC express functional A2A and A2B receptors. However, only stimulation of A2A, but not A2B, leads to EC barrier enhancement and this process is potentiated via the cAMP stimulating G-protein, G_s and the activation of the protein phosphatase, MLCP. Ongoing studies to define A2B receptor signaling in pulmonary EC will be important for our understanding of the adenosine signaling. Adenosine may be added to the growing list of key signaling molecules that regulate vascular function and homeostasis and to a very select list of agonists that promote integrity of the vascular bed. The characterization of this biologically active nucleoside in cell-cell communications may be critical to understanding the complexity of permeability, wound healing, and anti-inflammatory effects, as well as various other lung disorders.

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Fig. 1. Adenosine enhances HPAEC barrier function. (A) Dose-dependent response to adenosine on TER. HPAEC were challenged with increasing concentrations of adenosine (1–100 μ M). Data are representative of multiple independent experiments (minimum of three). (B) The protective effect of adenosine on thrombin-induced EC barrier dysfunction in HPAEC. In these experiments, HPAEC were either simultaneously treated with adenosine (50 μ M) and thrombin (100 nM) or adenosine was added to the cells after 30 min of thrombin treatment. Adenosine alone consistently elicits a significant increase in TER but adenosine and thrombin mix returns to the baseline control values but does not exhibit the 40% decline in TER below the control values observed in thrombin alone treatment. Furthermore, adenosine rapidly and consistently restores the HPAEC barrier integrity in cells stimulated previously with thrombin. Data are representative of three independent experiments. Results are presented mean $\pm$ SE and derived from three independent experiments (* $p < 0.05$ compared with control).

Fig. 2.

Effect of adenosine on the cell–cell junctional proteins in HPAEC. (A) Immunofluorescence studies of VE-cadherin. Cells were treated with 50 μ M adenosine for 30 minutes and were stained for VE-cadherin. Appreciably more VE-cadherin is recruited to the area of cell–cell junctions after adenosine treatment. Arrows indicate overlapping edges of neighboring cells. (B) Quantification of the surface area of the cell–cell interface. The percentage of total cell-surface area occupied by VE-cadherin-labeled cell–cell junctions was calculated for 20 cells in each group. The graph demonstrates that adenosine-induced a significant increase in cell–cell interface surface area as a percentage of total cell surface area ($*p < 0.05$ compared with control). The box and Whiskers plot show the means (lines at the box centers, 21.42% and 59.03% for control and adenosine-treated cells respectively). (C) Immunoblot analysis of the biotinylated (cell-surface) protein fractions isolated from the HPAEC challenged with 50 μ M adenosine. Elevated cell-surface levels of VE-cadherin confirm increased tightness of cell–cell contacts after adenosine stimulation. Results are presented mean $\pm$ SE and derived from four independent experiments ($n=4$).

Fig. 3. Effect of cytoskeletal alterations on adenosine-induced endothelial cell barrier protection. (A) HPAEC were pretreated with either vehicle or cytochalasin B for 30 min and then stimulated with 50 μ M adenosine in TER measurement assay. Actin depolymerization decreases TER and completely prevented the effect of adenosine on TER. (B) HPAEC were pretreated with either vehicle or the microtubule-disrupting agent, nocodazole for 30 min and then stimulated with 50 μ M adenosine. Disruption of microtubules decreases TER, but failed to alter adenosine-induced increases in TER. Results presented are mean $\pm$ SE and derived from three independent experiments.

Fig. 4.

(A) Expression of adenosine receptors (A1, A2A, A2B, and A3) mRNA in HPAEC and the effect of A2A and A2B receptor-specific agonists and antagonists on the HPAEC barrier function. Receptors mRNA expression was examined by Real-Time RT-PCR. Data was calculated relative to the internal housekeeping gene (18SrRNA) and are expressed as a fold change compared with the control $\pm$ SEM (n=4). (B) A2A receptor specific agonist, pDITC-APEC (100 nM) significantly increases the HPAEC TER response. (C) Pretreatment (10 min) of HPAEC with A2A receptors antagonists ZM 241385 and CGS15943 attenuated the adenosine-induced TER response to a large extent indicating the requirement of A2A receptors in EC TER increase. (D) Pretreatment (10 min) of A2B receptors antagonist, PSB 36, failed to attenuate the adenosine-induced TER increase suggesting that A2A, but not A2B receptors are involved in HPAEC TER increase.

Fig. 5. Studies on the depletion of A2A and A2B receptors using sequence-specific siRNAs. HPAEC were treated with indicated A2A or A2B receptor specific siRNA or non-specific siRNA for 48 hr and then challenged with 50 μ M adenosine in TER measurement assay. Depletion of A2A, but not A2B significantly attenuates the adenosine-induced TER increase. Receptors mRNA depletion was confirmed by RTPCR. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a loading control.

Fig. 6.

Effect of G-protein depletion on adenosine-induced TER increase. HPAEC were treated with indicated Gα isoform-specific siRNAs (Gαs, Gαq or Gαi2) or non-specific siRNA for 48 hr and the mRNA expression was detected by RT-PCR. HPRT was used as loading control. HPAEC were treated with indicated Gα specific siRNA or nonspecific siRNA and then challenged with 50 μM adenosine in TER measurement assay. Although depletion of Gαq or Gαi2 did not alter adenosine-induced TER increase, depletion with Gαs significantly attenuates the EC barrier enhancement property of adenosine indicating the involvement of cAMP-dependent signaling. Results are presented as mean ± SE and derived from three independent experiments.

Fig. 7.

(A) Effect of MYPT1 siRNA on adenosine-induced TER increase. HPAEC were treated with MYPT1 siRNA or non-specific siRNA for 48 hr and then challenged with 50 μ M adenosine in EC TER measurement assay. Depletion of MYPT1 significantly attenuates the effect of adenosine on TER. The expression of MYPT1 protein was detected by immunoblotting with MYPT1 antibody. β -tubulin was stained as loading control. (B) Inhibition of PP2A failed to alter the adenosine-induced increase in TER. HPAEC were pretreated with either vehicle or PP2A inhibitor fostriecin for 30 min, followed by stimulation with 50 μ M adenosine in EC TER measurement assay.